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AWARD NUMBER DAMD17-97-1-7142

TITLE: The Effect of MHC Class II Transactivator (CIITA) on the Growth and Metastasis of Breast Tumors

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REPORT DATE: June 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1998	Annual (1 Jun 97 - 31 May 98)

4. TITLE AND SUBTITLE

The Effect of MHC Class II Transactivator (CIITA) on the Growth and Metastasis of Breast Tumors

5. FUNDING NUMBERS

DAMD17-97-1-7142

6. AUTHOR(S)

Brian Martin

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research And Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

CIITA is a recently described protein that up regulates several different genes involved in the MHC class II antigen presentation pathway. I hypothesize that the *de novo* expression of molecules involved in the MHC class II antigen presentation pathway may render the tumor cells immunogenic and lead to tumor rejection. Also, preliminary data in our lab has revealed that, in addition to its effects on class II genes, CIITA is also able to induce MHC class I genes in tumor cell lines that have low or no expression of these molecules. Given that early studies have shown that *de novo* expression of syngeneic MHC class I genes in some tumors can induce tumor immunity and that approximately 33% of breast tumors have downmodulated class I expression, CIITA may be an effective molecule for the induction of tumor immunity via MHC class I and class II. The induction of tumor immunity using a global transactivator is a novel therapeutic strategy. I propose to accomplish these goals with two aims: 1) Determination of the genes induced by CIITA in breast carcinoma cell lines. 2) Determination of the effect of CIITA expression in breast carcinoma cell lines on *in vivo* growth, metastasis and vaccine efficacy.

14. SUBJECT TERMS

Breast Cancer

CIITA; MHC class II; MHC class I; immunotherapy.

15. NUMBER OF PAGES 16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Brian K. Mann 6/29/98

PI - Signature Date

Table of Contents

Introduction

MHC class II function, expression and effect on tumorigenesis. MHC class I and class II molecules are cell surface glycoproteins that are involved in the antigen presentation arm of the immune response. MHC class II molecules are composed of heterdimeric α and β chains. When these chains are synthesized in the endoplasmic reticulum (ER) they are bound by a related molecule termed invariant chain (Ii) (1). Ii prevents the class II heterodimer from binding to peptides in the ER (2,3) and provides signals necessary for proper trafficking (4). From the ER to the trans–golgi there is cleavage of Ii, leaving an Ii peptide (CLIP) binding MHC class II (5,6). The class II-CLIP complex then moves to a lysosomal compartment termed the MHC class II compartment or MIIC (7). There, the class II related molecules DMA and DMB facilitate the removal of the CLIP peptide and exchange with peptides derived from extracellular material (8-10). The class II peptide complex is then shunted to the cell surface where antigen is presented to CD4+ T lymphocytes.

Proteins in the class II pathway are generally restricted in expression to professional antigen presenting cells, although their expression can be induced in many other cells by a number of stimuli, the most potent being interferon(IFN)–γ (11-13). Most tumors lack expression of MHC class II molecules unless they have been derived from a tissue or cell type that normally expresses class II (14). A few experiments have sought to determine the effect of *de novo* class II expression on the growth characteristics of tumor cells. Our collaborator, Suzanne Ostrand-Rosenberg, has shown that transfection of syngeneic class II α and β chains alone is sufficient to induce lonγ–term tumor immunity in a sarcoma model (15-19). This immunogenicity is greatly enhanced with B7-1 expression (20-22). Similar findings have been shown with a neuroblastoma model transfected with human class II α and β chains (23). Interestingly, when the α and β chains are coexpressed with Ii, the protective effect of α–β alone is abrogated, leading to the proposal that class II proteins in the absence of Ii present endogenous tumor peptides while, in the presence of Ii, this activity is blocked (24). However, without expression of the DM molecules, it is unlikely that the cells were able to present typical class II peptides because of the blockade of the binding groove by CLIP. Another report demonstrated that, in a tumor with little or no class II and class I expression, class II in concert with class I was able to prevent tumor growth and induced long term immunity (25). In this model class II α–β expression alone was insufficient. Finally, a recent report by Panelli, et al., demonstrates that IFN–γ transfection of EMT6 mouse tumor cells increases their immunogenicity (26). IFN–γ expression increased expression of class II, accompanied by expression of CIITA, showing that CIITA may play a significant role in the induction of immunity in a breast cancer model. An area remaining to be studied is the effect of class II expression on tumorigenicity in the presence of the other MHC class II antigen presentation molecules.

MHC class I function, expression and effect on tumorigenesis. MHC class I antigen presentation, in general, functions by presentation of cytosol derived peptides to CD8+ T lymphocytes. These peptides are produced via a cytosolic 20s proteasome that processes proteins produced in the cytoplasm (27). The resultant peptides are moved into the ER using specific peptide transporters, TAP (transporter associated with antigen

presentation)1 and TAP2 (28-30). Class I heavy chains are synthesized in the ER in connection with a chaperone called calnexin (31-33). After association with β2-microglobulin, the class I protein is competent to bind to peptide. When the peptide is loaded, calnexin dissociates and the molecule is competent for egress from the ER. After being shuttled through the Golgi to the cell surface by the exocytic pathway, the class I/peptide complex is presented for recognition by CD8+ T lymphocytes, that are then able to induce cytotoxicity. Class I molecules have a generally ubiquitous expression pattern (reviewed in (34).

The effect of MHC class I expression on tumor cells that lack that expression has been the topic of much more study than for class II (reviewed in (35,36). Many studies have shown that several types of cancer have decreased class I expression relative to their tissue of origin (reviewed in (14,35). This includes several studies of human breast cancer cells that have shown 33% have decreased class I expression relative to normal breast tissue (37-39). Downregulation of MHC class I genes by various mechanisms (such as decreased heavy chain transcription, decreased levels of TAP and/or proteasome subunits) may be a primary mechanism of escape from the immune system (reviewed in (14,35), but there may be several other explanations for this phenomenon (14). As for MHC class II, the effect of a transcriptional regulator of class I on tumor immunity has not been studied.

Several landmark studies in the mid-1980s demonstrated that expression of MHC class I heavy chain molecules in cells that lack surface class I could lead to abrogation of tumor growth, loss of metastatic capability and/or induction of protective immunity (40-42). In breast cancer, it has been found that lack of surface class I expression correlated with a more malignant phenotype (37). Two early studies demonstrated a poorer prognosis for breast cancer patients with low class I expression on their tumors (38,39). These studies suggest that class I and class II expression are very important determinants in breast cancer immunogenicity.

MHC class II transactivator (CIITA). CIITA is a master regulator of class II MHC gene expression. Patients with a deficiency in class II expression, referred to as bare lymphocyte syndrome (BLS), suffer from severe immunodeficiency. Complementation cloning of the defective gene in a subgroup of BLS led to the identification of CIITA (43). This novel gene was analyzed and found to be a global regulator of the class II genes. *de novo* expression of CIITA facilitates expression of all the classical class II α and β chains (DR, DP, DQ), Ii and the DM genes (44-47). A N-terminal domain in the protein has been shown to be an activation sequence when fused to the Gal-4 DNA-binding site but CIITA has not been demonstrated to have intrinsic DNA-binding activity (48,49). The CIITA gene has been knocked out in mice via homologous recombination techniques and mice with the defect have cells reminiscent those of BLS patients (50). These studies demonstrate CIITA to be a global transactivator of the class II antigen presentation pathway. In addition, the applicant has recently demonstrated that CIITA is able to induce expression of class I MHC in cancer cells that have low or no expression of these molecules (see Methods for preliminary data).

The use of CIITA to induce immunity against breast cancer cells represents an important and novel concept. Although many other studies have sought to determine the

effect of α and β chains of class II alone on tumorigenicity, those studies were done without the accessory molecules necessary for proper class II antigen presentation. CIITA is able to upregulate all known molecules involved in class II antigen presentation. In tumors without class I MHC, CIITA is able to induce its expression. The use of such a global transactivator as an immunotherapeutic molecule has not been documented for breast or other cancers.

Hypothesis/Purpose

CIITA has been shown in many systems to induce several genes involved in the MHC class II antigen presentation pathway (44-47). In some instances, *de novo* expression of CIITA has lead to enhanced antigen presenting cell (APC) function. I have recently shown that, in addition to class II molecules, CIITA is able to induce MHC class I surface expression in cells deficient in expression of these molecules. I hypothesize that *de novo* expression of CIITA in breast tumor cells will upregulate class II genes, and in the case of cells with low or no expression of class I, class I genes. The expression of these molecules may induce an immune response against these cells, affecting growth, metastasis, and vaccine efficacy. Should this not induce a response, the coexpression of costimulatory molecules may be necessary for immune response induction. I hypothesize that CIITA expression has the potential to be a novel mechanism for induction of immunity to breast cancer.

Body

Experimental Methods

Cells: The MT901 Balb/c mammary carcinoma was obtained from Dr. Larry Turka (U. Penn.). The 4T1 mammary tumor was obtained from Dr. Suzanne Ostrand-Rosenberg (U. Maryland). The EMT6.8 mammary carcinoma was obtained from Dr. John Frelinger (U. Rochester). All cells were maintained in DMEM (Gibco-BRL, Gaithersberg, MD) with 7% fetal bovine serum (FBS) (Gibco-BRL).

Transduction: The EcoRI fragment of the FLAG.CIITA8 construct was cloned into the EcoRI site of the LXSNb retroviral plasmid (51,52). The construction of the CIITA mutant plasmids is described elsewhere (52). Production of retrovirus was as previously described (51). Briefly, plasmid DNA (either control vector or CIITA containing vector) was transfected into the PA317 helper cell line via calcium phosphate precipitation and the following day the media were changed. 48 hours after transfection, the supernatant was collected, sterilized by filtration and stored for later use at -70°C.

Cells were seeded on plates at 2.5 x 10^5 cells per 35 mm plate on the day before transduction. Viral supernatant (250 μl) with 8 μg/ml polybrene was added to 1 x 10^5 cells and incubated for 2 hours at 37°C. After incubation the viral supernatant was aspirated and replaced with fresh growth medium. Two days after transduction, the cells were passaged 1:20 and placed in selection media (400 μg/ml G418). The resultant polyclonal population was then analyzed for class II expression.

Flow Cytometry Analysis of MHC Class I and Class II Expression: The antibodies used for these studies were kindly provided by Dr. J.F. Frelinger; M1/42 (rat anti-mouse H-2), 34-7-23s (anti H-2 K^d/D^d), BP1072.2 (anti I-Eβ/I-Aβ, reactive with haplotypes d,b,p,q,u,j), 7-16/17 (anti I, reactive with haplotypes p,b,k,q,r,s,j). Secondary

antibodies used were goat anti-mouse IgG-FITC conjugate (Pharmingen) and goat anti-rat IgG FITC (Sigma, St. Louis, MO).

For flow cytometry, cells in log growth phase were harvested and washed twice with 1xPBS containing 0.1% sodium azide. The cells were resuspended at 1 x 10^7 cells per ml and 100 µl used for each sample. The cells were incubated for 30 minutes with diluted primary antibody (20 µl per sample). The cells were washed three times with 1XPBS-NaAzide and then incubated for 20 minutes in diluted secondary antibody (20 µl). The cells were washed three times with 1XPBS-NaAzide. The cells were either analyzed immediately or fixed in 2% paraformaldehyde and stored for less than one week for analysis.

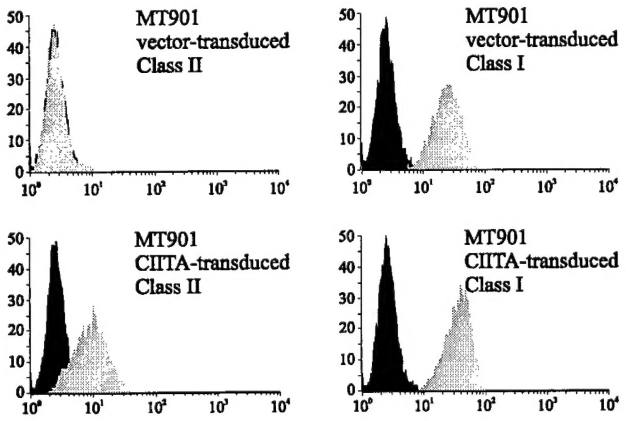
Flow cytometry was performed on a Becton-Dickinson FACScan (San Jose, CA) using Cyclops software (Cytomation, Fort Collins, CO). 5000 cells were analyzed for each sample.

Mouse Experiments: Various cells doses (as indicated by the literature) were injected into mice. The minimal tumor dose (MTD) was determined as the lowest tumor number in which all mice grew tumors. For CIITA studies in the MT901 tumor system, CIITA transduced (LCIITASN) and vector transduced (LXSN) cells were injected at 10X the MTD (1 X 10^5 cells per mouse) subcutaneously.

Results

The MT901 lung carcinoma was transduced with CIITA and the surface expression patterns of MHC class I and class II were analyzed. As shown in Figure 1, CIITA increased the expression of both class II and class I in this cell line.

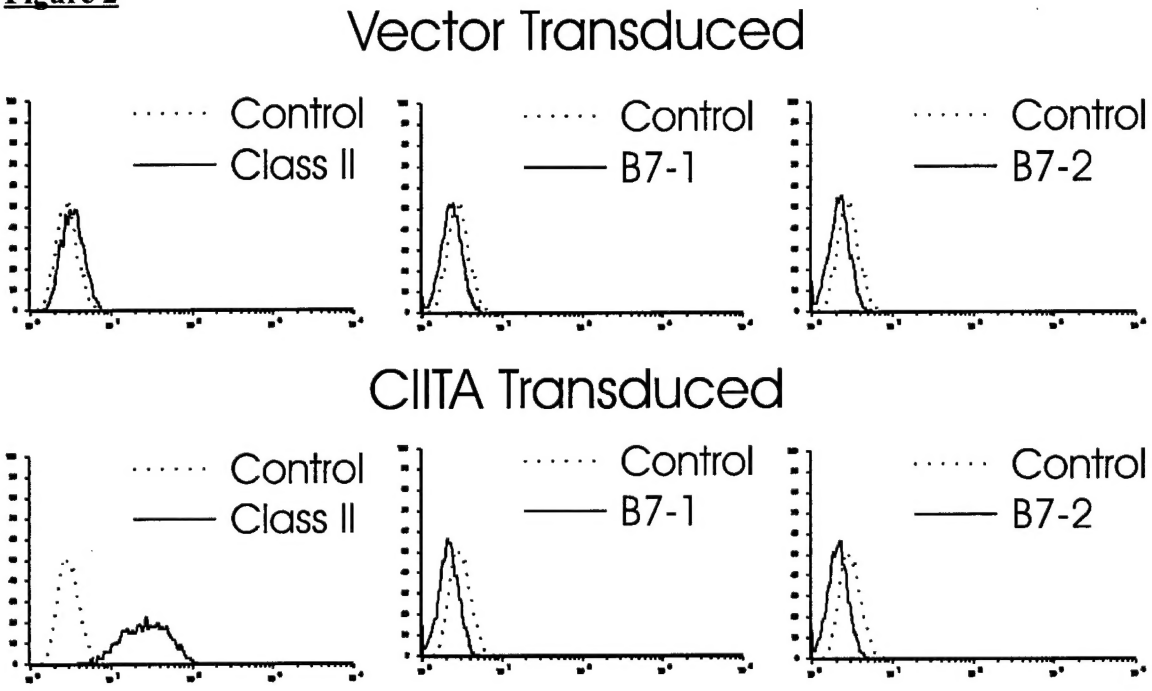
Figure 1



CIITA expression in this cell line increased MHC class II expression approximately 10 fold. Additionally, unlike cell lines derived from other tissue types (53), the level of expression of MHC class I actually increases slightly under the influence of CIITA. In other cell lines with moderate to high basal expression of MHC class I, CIITA does not affect the level of class I. These results suggest that CIITA may influence the immunogenicity and tumorigenicity of MT901 on two levels, MHC class I and/or MHC class II.

We have examined the effect of CIITA transduction on the expression pattern of the costimulatory molecules B7-1 and B7-2 and have found that in no case does expression of CIITA induce expression of these genes in MT901 or in other tumor models systems tested in our laboratory (see Figure 2).

Figure 2

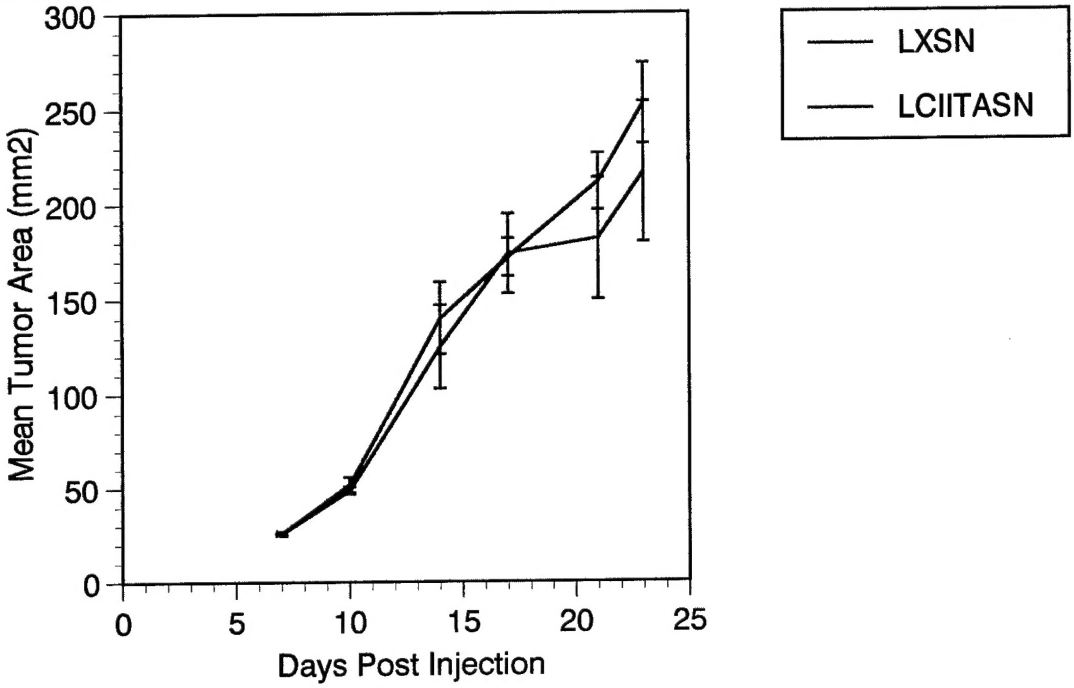


In the 4T1 mammary tumor, CIITA expression had an unexpected effect. When the cells were transduced with control retroviral expression vector, approximately 1000 colonies developed. However, when these cells were transduced with CIITA retrovirus, only approximately 10 colonies developed. None of the colonies that grew up appreared to have an intact CIITA gene (data not shown). Transfection (rather than transduction) was also attempted on this cell line with CIITA in the pcDNA3 vector. Once again there were approximately 100 fold fewer colonies on the CIITA plate as opposed to the control plate. In all other cells lines transduced with CIITA retrovirus, there has never been such a large discrepancy between control versus CIITA infected cells. These data strongly suggest that CIITA is acting directly to kill the cells, and preliminary evidence implicates an apoptotic mechanism. We are currently developing an inducible expression system with CIITA in order to determine the mechanism of this directly killing by CIITA.

Due to the fact that CIITA kills 4T1 and as proposed in the Fellowship Training Proposal, we have obtained a third breast cancer cell line (EMT6.8 from Dr. John Frelinger) to increase the number of lines to be assayed for the CIITA effect. However, this line has yet to be transduced with CIITA.

We have proceeded with preliminary experiments designed to test the effect of CIITA expression on the *in vivo* growth and immunogenicity of mammary tumors. In collaboration with Dr. Larry Turka and his graduate student Steven Eck, we have begun testing in this model system. In the following experiment, mice were injected with 10X the MTD and monitored for tumor growth (see Figure 3).

Figure 3



No difference was observed between the CIITA versus control transduced MT901 tumor cells. These data suggest that CIITA, in and of itself, may have little effect on primary tumorigenicity in the MT901 tumor system.

Discussion

The results with the MT901 mammary tumor line indicate that CIITA effectively induces the expression of MHC class II genes and proteins. In addition, CIITA also enhances the expression of MHC class I. In our previous report, we had shown that CIITA effectively induces the expression of class I in cell lines with low or no expression of class I, but was ineffective in cells with moderate to high class I expression (53). MT901 has moderate basal levels of class I (see figure 1), the levels of which are increased by CIITA expression. This suggests that CIITA may increase immunogenicity through class I and/or class II MHC. However, it must also be kept in mind that in the absence of costimulatory molecules, increased expression of class I and/or class II may actually induce an anergic state.

Further efforts to identify other genes and proteins have not yielded any additional candidates. Neither B7-1 nor B7-2 are induced in any tumor lines thus far tested. This would also seem to indicate that, should CIITA in and of itself not be sufficient for enhancement of tumorigenicity or immunogenicity, other means of providing costimulation are required. Vectors that are capable of delivering both CIITA and B7-1 or B7-2 simultaneously are currently under development. This consists of a bicistronic vector utilizing an internal ribosome entry site (IRES).

The finding that CIITA induces an cytotoxic effect in the 4T1 mammary carcinoma are intriguing. If, in some instances, CIITA is able to directly stimulate apoptosis or an otherwise necrotic phenotype in tumor cells, this would indicate its utility as a direct cytotoxic mediator. Two protocols are currently under development to test this theory. First, a Tet inducible system for CIITA expression is being worked out in the laboratory. This consists of the CIITA gene in the antisense orientation in a retroviral vector. This gene is under the control of the Tet operon, meaning that in the presence of tetracycline, CIITA is off. What little background expression there is should be controlled by antisense from the LTR promoter from the retrovirus. When you remove tetracycline, CIITA is now expressed. This allows an entire population of cells in which we can directly observe the cytotoxic effect of CIITA expression in the 4T1 system.

The second system is the development of high titer retrovirus. In collaboration with Dr. John Olsen here at UNC, I have used a VSV-G based system that allows the production of viral titers of greater than 10^8 colony forming units (CFU) per ml. This allows the infection of nearly 100% of the cells being tested. It also allows *in vivo* injection of retrovirus with very high titers to test the efficacy of CIITA therapy. To date, I have the CIITA construct completed and am now proceeding to make the retrovirus itself.

Due to the fact that we now have only one tumor system to test in vivo (MT901) we have a procured the EMT6.8 mammary tumor and are proceeding to test this model.

Our preliminary tests with tumorigenicity assays indicate that there is no effect of CIITA expression on the primary tumor growth of MT901. While not what we have hoped for, this result is not entirely unexpected. It is long held dogma that immune stimulation via class I or class II MHC in the absence of costimulation can lead to an anergic state. This suggests that the combination of CIITA with costimulatory molecules may be more efficacious. However, it must also be kept in mind that in a clinical setting, live tumors would never be injected into a patient. Instead, modified tumors are irradiated and then injected into the patient to stimulate a systemic immune response against metastatic tumor. This is more closely approximated by mouse studies utilizing irradiated tumors followed by wild type tumor challenge. These studies are currently being initiated. An additional possibility is that there are other molecules necessary for effective MHC class II antigen processing and presentation that are not induced by CIITA. For instance, Mach et al., have found that a cathepsin protease family member is required for effective antigen presentation in a melanoma model (54), however, we have found that CIITA induces effective antigen presentation in a sarcoma cell line (55). This suggests that the efficacy of CIITA in inducing effective antigen processing and presentation may be specific in a given tumor model system.

Recommendations with regard to Statement of Work

Nearly all aspects of Year 1 have been completed. All mammary tumor model systems have been procured. All (with the exception of EMT6.8) have been tranduced with CIITA. The effect of CIITA on class I and class II MHC expression has been evaluated on these lines. Other genes (namely, the costimlatory molecules B7-1 and

B7-2) have been examined and shown not to be induced by CIITA. Finally, the MTD for both MT901 and EMT6.8 have been determined. In addition, the effect of CIITA on MT901 immunogenicity has been tested as well (a Year 2 Statement of Work project). These results show that the work as proposed is precisely on schedule.

Conclusions

The results presented herein demonstrate that the MHC class II transactivator (CIITA) is able to efficiently upregulate MHC class II and to a lesser degree MHC class I in a mouse mammary tumor model, MT901. CIITA does not, however, induce the costimulatory molecules B7-1 or B7-2. Introduction of CIITA into MT901 does not appear to influence the primary tumorigenicity of this model, suggesting the requirement for additional costimulation. CIITA has a direct cytotoxic effect in the 4T1 mammary carcinoma, suggesting that in some model systems it may be efficacious in the induction cell death, and so primary tumor therapy. Collectively, these data suggest further studies in order to determine the utility of CIITA in human breast cancer tumor therapy.

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